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GRANT NUMBER DAMD17-94-J-4349

TITLE: Use of Cytokines to Prevent Breast Cancer Growth and Progression

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REPORT DATE: August 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1996	Annual (1 Aug 95 - 31 Jul 96)

4. TITLE AND SUBTITLE
Use of Cytokines to Prevent Breast Cancer Growth and Progression

5. FUNDING NUMBERS
DAMD17-94-J-4349

6. AUTHOR(S)
Phillip M. Grimley, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry M. Jackson Foundation
For the Advancement of Military Medicine
Rockville, Maryland 20852

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Goals of this project include: (1) characterization of a novel transcriptional knock out (TKO) factor which negatively regulates signal activation by type I interferons (IFN-α/β) in breast cancer cells; and (2) analysis of signal interactions of IFN-α/β and prolactin (PRL) in breast cancer cells. (1) Improved purification of TKO from suspension cultures of human cancer cells has been achieved: a single functionally active polypeptide of ~20 kDa has been isolated. Approximately 300 ng of TKO is obtained per batch of ~70 x 10^{10} cells, and samples are in process for proteolytic digestion and mass spectrometric analysis. (2) PRL interactions with IFN-α/β were explored in differentiated (T47D, MCF-7) and undifferentiated (BT-20) cells. IFN-α/β predictably activated Jak1 and Tyk2 protein tyrosine kinases (pTK) involved in Stat2-Stat1 phosphorylations. Whereas PRL typically activates Jak2, it also activated Jak1 and Tyk2. While differential regulation of pTK activity may be one control mechanism influencing PRL gene transactivation signals; there nevertheless was no evidence of antagonism between IFN-α/β and PRL at the level of Stat2-Stat1 activation. Indeed, additive Stat activation was found in T47D and MCF-7 cells. While PRL invariably activated Stat1, activation of Stat3 and Stat5 proved selective. Current work is probing the mechanisms and consequences of such signal diversity.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	18
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Phil M. Trunky 11-15-96

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(5) INTRODUCTION

Treatment decisions for patients with disseminated breast cancer can pose a difficult problem for oncologists due to the morbidity associated with conventional genotoxic agents which are mutagenic and increase the risk for subsequent development of leukemia[1]. This can be particularly onerous when a stage II breast cancer is negative for estrogen receptors and the patient is under age 50 years. Interferons (IFNs) are natural cytokines with antiproliferative properties. Type I interferons (IFN-α/β) can block entry of cancer cells into S-phase of the cell-division cycle[2] and act to cause cell differentiation[3-5]. Potential benefits of IFN antiproliferative therapy include an avoidance of permanent damage to normal non-proliferating tissues, mitigation of cytotoxic side-effects and elimination of the hazard of delayed carcinogenicity. IFN-α has been tested repeatedly in preclinical models[6-8] and in phase I/II clinical trials[9, 10]; and it is currently included as adjuvant therapy in some treatment protocols[11].

The current project encompasses two major goals: (1) to purify and characterize a novel transcriptional knock out (TKO) polypeptide which we have identified in breast cancer cells and which competitively interrupts the ISGF3 signaling pathway for IFN-α/β; and (2) to explore and define the molecular interactions of IFN-α/β, which act as growth inhibitors, with prolactin (PRL) which is as a potential growth promoter for breast cancer[12, 13].

(1) TKO: Expression of genes leading to the principal biologic actions of IFN-α/β, including antiproliferative activity, depends upon the transcriptional function of cytoplasmic Stat proteins which serve in a dual capacity as signal transducers and activators or transcription[14-18]. In the case of type I interferons, signal transduction is initiated by dimerization and transphosphorylations of type I IFN receptors[15, 17]. This signal initiation is intimately associated with autophosphorylation and transphosphorylations of reciprocally interdependent Janus tyrosine kinases Jak1 and Tyk2[14, 17]. Associated tyrosine transphosphorylations of Stat proteins predispose to the formation of Stat homodimers or heterodimers which translocate to the nucleus, bind to highly conserved promoter regions upstream of IFN stimulated genes, and enhance or initiate transcription[15, 16]. Relevant nucleotide elements within upstream promoter regions include IR[18, 19] and Fcγ GAS[20] which bind Stat1 dimers and an interferon sensitive response element (ISRE) which uniquely binds a multimeric complex (ISGF3)[14, 16, 17]. The ISGF3 holocomplex incorporates Stat2, Stat1 and a 48kDa DNA-binding protein (ISGF3γ) in the *myb* family[14, 16, 17, 21]. The ISRE acts as a positive transcriptional regulator in a large proportion of known interferon responsive genes[16]. The intranuclear translocation of ISGF3 and maintenance of gene transactivation by IFN has been directly correlated with the Stat phosphorylation status[22].
Activation of Stat1 by a IFN-α/β depends upon the activation of Stat2[23]. IFN-α/β can not generate a Stat1 signal in mutant cells which lack either Stat2 or the transphosphorylating Janus kinases Jak1 or Tyk2[23-25]. *In vivo*, deficiencies of Stat1 tyrosine phosphorylation have been linked to a loss of IFN stimulated antimicrobial defenses[26, 27]; and a physiologic specificity of Stat1 proteins in the biologic responses to IFN in mice was clearly shown by targeted disruption of Stat1 gene function[27].

In work preliminary to this project, we showed that the signal transduction pathway for IFN-α can be selectively interrupted by a negative regulator of transcriptional activation detected

in the cytoplasm of malignant cells[28]. This negative regulator was ultimately identified as a polypeptide with a molecular weight of ca. 20 kDa and is designated as *transcriptional knock out* factor or *TKO*. The mechanism of TKO negative regulatory action was explored and related to inhibition of DNA binding by the 48 kDa subunit in the ISGF3 transcriptional holocomplex. The 48 kDa polypeptide is structurally homologous to a family of IFN regulatory factors (IRF) which include a DNA-binding tumor suppressor protein IRF-1[18], an oncoprotein IRF-2, and a gene repressor ICSBP[21].

TKO activity is detected in breast and cervical cancer cell extracts by means of electrophoretic mobility gel shift assays (EMSA) using specific oligonucleotide probes to represent the ISRE[28]. Although both the Stat2-Stat1 heterodimer and the 48 kDa polypeptide of ISGF3 each individually are capable of binding to an ISRE nucleotide sequence, the binding affinity is ~25-fold increased when subunits are combined in the ISGF3 holocomplex. By actively competing with the 48 kDa polypeptide (ISGF3γ), TKO inhibits binding to a ^{32}P-oligonucleotide ISRE probe. In EMSA the TKO inhibition results in absence of a band shift or a mobility "supershift". TKO inhibitory activity also can be detected with a probe for the IR promoter region of the IRF-1 gene by EMSA[28]. Using EMSA, significant TKO activity thus was found in cytoplasmic extracts from two lines of human cells originating from malignant breast lesions (Fig. 2 in Progress Report of 1995).

(2) Interferon-prolactin interactions: In addition to the interferons and interleukin cytokines, certain polypeptide hormones or growth factors, including erythropoietin, growth hormones, and prolactin (PRL) similarly activate gene expression through the Janus kinase / Stat pathway [14, 29-31]. PRL is a principal mammotrophic growth factor[32] and 40-70% of human tumor biopsies are positive for PRL receptors[33]. Intriguingly, PRL can serve either as a mammary epithelium growth promoter[12, 32], or as a cytostatic differentiation agent[34, 35]. Many tumor-derived cell lines express increased levels of PRL receptors and can proliferate in response to PRL *in vitro* (14-18)[36-38]. Although PRL normally originates from pituitary gland acidophil cells, recent work indicates that it can be endogenously produced in human breast cancers[13]. Thus endogenous PRL could be exercising some autocrine or paracrine role in modulating the antiproliferative response of breast cancer cells to IFN-α/β therapy. In present work we have explored the interactions of IFN-α/β and of PRL in three breast cancer cell lines representing differentiated (T47D, MCF-7) and undifferentiated (BT-20) phenotypes.

(6) BODY:

TKO purification and mass spectrometric analysis: A cervical cancer cell line, C33a has proven optimal for isolation of TKO, since it can be grown rapidly in mass suspension cultures. Using an extraction procedure refined during this project, we were able to obtain ~300 ng of highly purified product from each batch of ~70 liters of C33a cells grown in multi-liter spinner cultures at a density of ~1 x 10^6 cells/ml. Purification of protein with TKO activity was refined under the direction of collaborator E. Petricoin. Cells were dounce homogenized in Buffer A (20 mM KCl; 10 mM HEPES @ pH7; 5 mM dithiothreitol) with 20% glycerol and 0.1% NP40. Cytoplasmic extract is obtained by centrifugation of the whole cell lysate at ~8,000*g* for 30 min. The supernatant is loaded over a heparin-sepharose resin. The flow-through and two column washes are collected and pooled, then loaded onto a hydroxylapatite column. The active fraction elutes in a 0.2-0.4 M sodium phosphate gradient. It is brought to 35% ammonium sulfate final concentration and spun at 8,000*g* for 30 minutes. A detergent layer from the top of the tube is collected and further purified by loading onto a phenyl-Sepharose hydrophobic interaction column equilibrated with 35% ammonium sulfate/Buffer A without detergent. The flow-through material is collected and applied to a T-butyl hydrophobic interaction C column at 35% ammonium sulfate. This further depletes non-specific proteins before final concentration on an hydroxylapatite column. Functional quality of product was verified by EMSA using recombinant 48 kDa protein (p48): TKO inhibits binding of the p48 to a ^{32}P-oligonucleotide representing the ISRE sequence of the interferon stimulated gene ISG54K. Specific activity, in EMSA assay, ultimately was increased >10^4 X (based upon relative protein concentrations in crude and purified extracts). An active TKO fraction similarly was purified from extracts of ZR75-1 breast cancer cells. The TKO polypeptide was resolved by two-dimensional gel electrophoresis (2D-gel). Fig. 3 in the previous Progress Report compared the electrophoretic mobility of TKO polypeptide(s) in the ZR-75-1 breast cancer cells and C33a cells in two dimensional gel electrophoretograms (2D-gels). The resolved spot with specific TKO activity was identified by a procedure which depleted TKO from the column purified extracts. Product was exposed to a synthetic N-terminal fragment (125 AA) of the 48 kDa ligand prior to the 2D-gel procedure. This significantly diminished silver or Coomassie blue staining intensity of the assumed TKO spots as compared to other gel zones. By way of confirmation, EMSA showed that the supernatant had retained the competitive inhibitory activity for DNA binding by ISGF3. Thus far, we have made 8 successful batches, out of a total of 12 attempts during which culture conditions were optimized. During these efforts we overcame of number of technical problems which led to dissipation of useful product in terminal stages of isolation.

Maximum column chromatographic and two dimensional electrophoretic purification of the TKO polypeptide has been combined with an improved immunoblot transfer to facilitate direct spectrometric analysis. Initial purified samples were sent to the laboratories of Dr. Donald Hunt at the University of Virginia Department of Chemistry or Dr. Lois Epstein at the University of California in San Francisco. Those efforts were not successful due either to quantitative sample insufficiency, a qualitative problem (amino-terminal blocked), equipment technical problems or personnel transfers in the collaborating facilities. A collaboration was therefore developed with the laboratory of Dr. Ruedi Aebersold in the Department of Microbiology at the University of Washington in Seattle. This laboratory is proficient in

analyses of minimal samples using the techniques tryptic digestion and electrospray ionization mass spectrometry[39, 40]. The method of transfer of the resolved TKO from 2D-gels has proven critical. Dr. Aebersold recommended staining of the 2D-gel with amido black to identify the TKO prior to transfer to nitrocellulose membrane. The procedure previously used unstained gels and transfer to PVDF membranes. The nitrocellulose-transferred TKO was washed in HPLC grade water, and wet frozen at -70°C for shipping in dry ice.

Successful and reproducible analysis of two separate batch samples was finally achieved by Dr. Steve Gygi in the Aebersold laboratory. Dr. Gygi was able to analyze amino acid sequences of nine main peptide fragments obtained by tryptic digestion. Material analyzed in each of these runs was from 2D-gel spots of functional TKO as indicated by an arrow in Fig. 3 of the 1995 Annual Progress Report. Analysis of the amino acid sequences has shown a region rich in hydrophobic amino acids consistent with a membrane or protein binding function. Based upon the sequence data we synthesized oligopeptides representing two highly charged hydrophilic fragments including the C-terminal fragment of the putative TKO molecule. Rabbits have been immunized and immune sera with polyclonal antibodies soon will be available to begin experimental testing of utility for functional inactivation of native TKO and detection of denatured TKO by immunoprecipitation and Western blotting. Depending upon the antibody quality, antisera could be used to assist in purification of native protein for whole protein mouse immunizations and monoclonal antibody production. We plan to generate monospecific antibodies by mouse intranodal injections; since microgram amounts of purified protein may be needed for this approach to succeed. Immune sera will be screened in EMSA for functional neutralization or supershift activities using recombinant p48 protein. Antibodies also will be tested in immunofluorescence or immunoperoxidase procedures with TKO positive cells from the ZR75-1 breast cancer line[41]. Tests with samples of other breast cancer cells lines, frozen biopsy tissues or samples of deparaffinized surgical biopsies, or aspiration cytology samples will follow.

Since TKO can block transcriptional activation of IRF-1 it can impede growth regulation and apoptosis. Identification of TKO in human breast cancers therefore has significant potential both as a therapeutic variable and prognostic indicator. Tissue identification will depend upon the technical capacity to screen patient samples. While TKO function could in principle be tested directly in EMSA assays of fresh tissue, this approach would be practically constrained by uncertainties in sampling of early lesions and diagnostic imperatives restricting the volume of tissue available for destructive analysis. Therefore, our ultimate goal is to develop means of TKO polypeptide or mRNA identification in tissue sections. In principle, the latter could be achieved with an anti-sense riboprobe based upon a cDNA sequence.

Analyses of interaction of PRL and IFN-α on Jak/Stat signaling in human breast cancer cells: Three PRL-responsive human breast carcinoma lines (T47D, MCF-7 and BT-20) were tested for Stat activation by IFN-α/β or PRL. Results indicated that signalling might be cell dependent both at the levels of Janus kinase and Stat activation. IFN-α/β had an expected effect in activating Jak1 and Tyk2 protein kinases involved in Stat2-Stat1 tyrosine phosphorylations, similar to results with other cell types. Results with PRL differed. In previous work with lysates from pre-T, pre-B or myeloid cell lines it was shown that Jak2 is the kinase primarily involved in transphosphorylation of Stat1 after PRL treatment (Fig. 1, panel A). In the breast

cancer cells a novel observation was that PRL also activated Jak1 and Tyk2. Results are compared in Fig. 1, panel B and indicate differences in intensity of response. Thus, pleiotropic regulation at the level of Jak activation may be one control mechanism governing the outcome of PRL receptor activation. Preliminary characterization of the effect of PRL on Jak1, Jak2 and Tyk2 in T47D cells showed that the three enzymes had comparable activation kinetics, peaking at 2.5-5 min (Fig. 2, panel A). Concentration responses also were similar, with EC_{50}-values of 3-10 nM PRL (Fig. 2, panel B).

PRL also activated Stat1 in each cell line (Fig. 3). This probably reflected the common Jak2 activation, since this particular kinase has been proposed to transphosphorylate Stat1 directly[17] and was the only invariably activated kinase in all three cell lines. The activation of Stat3 and Stat5 proved to be more cell dependent (Fig. 4). The activation of Stat5b shown in fig. 4 is of particular interest, since it has been shown to be involved in sustained proliferation of erythroid progenitors[42]. Since Stat proteins bind with partially overlapping specificity to regulatory regions of genes, current work aims to further identify and elucidate mechanisms and consequences of such signal diversity. Despite differences in the growth regulatory actions of IFN-α/β and PRL, no evidence of antagonism between IFN-α/β and PRL thus far has been detected at the level of the Stat2-Stat1 activation which is essential to the biological action of IFN-α/β. Indeed Fig. 3 shows an additive effect on Stat1-Stat2 tyrosine phosphorylation in T47D cells treated with IFN-α+PRL and MCF-7 cells treated with IFN-β+PRL for 15 min. We note that in highly differentiated MCF-7 cells, PRL, but not IFN-α, induced early Stat1 tyrosine phosphorylation as determined by immunoprecipitation with anti-Stat1 and phosphotyrosine immunoblotting; whereas, in well differentiated T47D cells PRL and IFN-α/β each induced comparable Stat1 tyrosine phosphorylation. In estrogen-receptor-negative and morphologically less differentiated BT-20 cells, IFN-α induced marked phosphorylation of Stat1 and Stat2, whereas PRL had only a minimal effect on Stat1. Interestingly, pretreatment of T47D or MCF-7 cells with IFN-γ for 24 hr served to enhance the PRL effect on phosphorylation of Stat1, possibly due to stimulation of Jak2 phosphorylation.

Based on knowledge from other related cytokine receptors and previous observation of selective Jak2 activation by PRL (see Fig. 1), it will now be of major significance to determine how PRL receptors couple to so many different Jak proteins in the same cell and to formulate the basis for differential activation of Stats. The prevailing view has been that individual receptor proteins have strong preference towards one Jak enzyme[43]. The common roles of Jak and Stat signaling elements in the signal transduction pathways of PRL and IFN-α/β and their biologic impact at the level of malignant cell proliferation needs further investigation. Future work must involve EMSA quantitation of Stat functional capacity by EMSA with [^{32}P]-oligonucleotide probes representing the ISRE or the gamma response region (GRR) enhancer elements of genes stimulated by IFN-α (ISRE and GRR) or PRL (GRR only). Proliferation status of the cells will be being monitored by cell cycle analyses, including flow cytometric analyses and immunoblot analyses of Rb phosphorylation, cyclin E and cyclin-dependent CDK or CDC kinase activities.

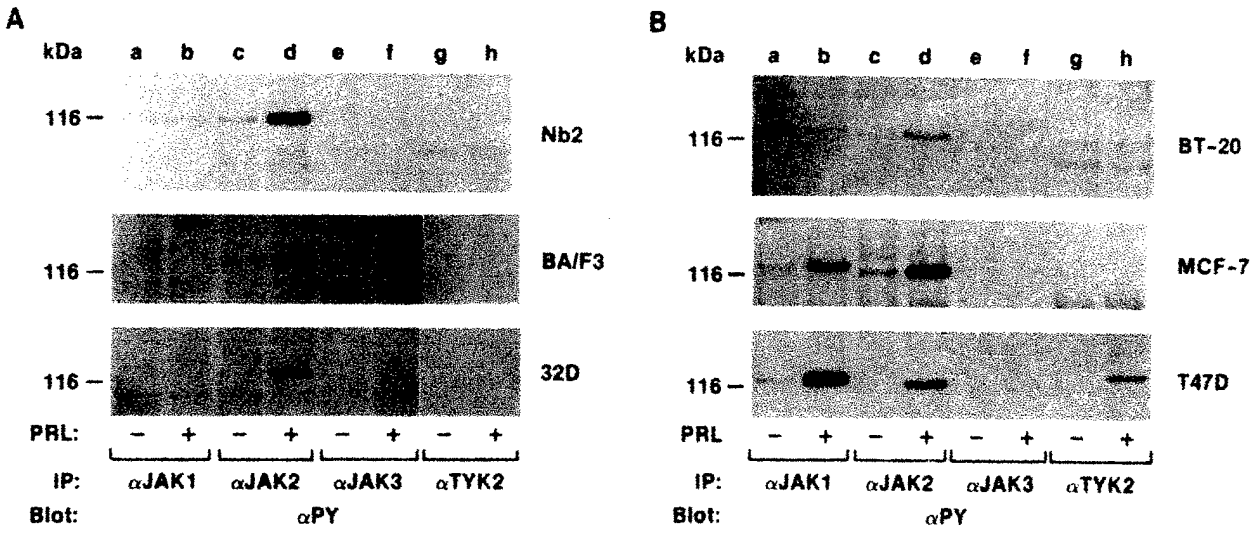

Fig. 1 PRL induces tyrosine phosphorylation of multiple Jak tyrosine kinases in a cell-dependent manner: Antiphosphotyrosine (αPY) immunoblots of immunoprecipitated (IP) Jak proteins (αJak1, αJak2, αJak3 and αTyk2) from lysates of pre-T lymphoid Nb2 cells, pro-B lymphoid BA/F3-PRLR cells (BA/F3), and myeloid 32D-PRLR cells (32D) (Panel A), and mammary epithelial cell lines BT-20, MCF-7 and T47D (Panel B). The cells had been incubated with (+) or without (-) 100 nM PRL for 5 min at 37° C. The 116 kDa molecular weight markers are indicated.

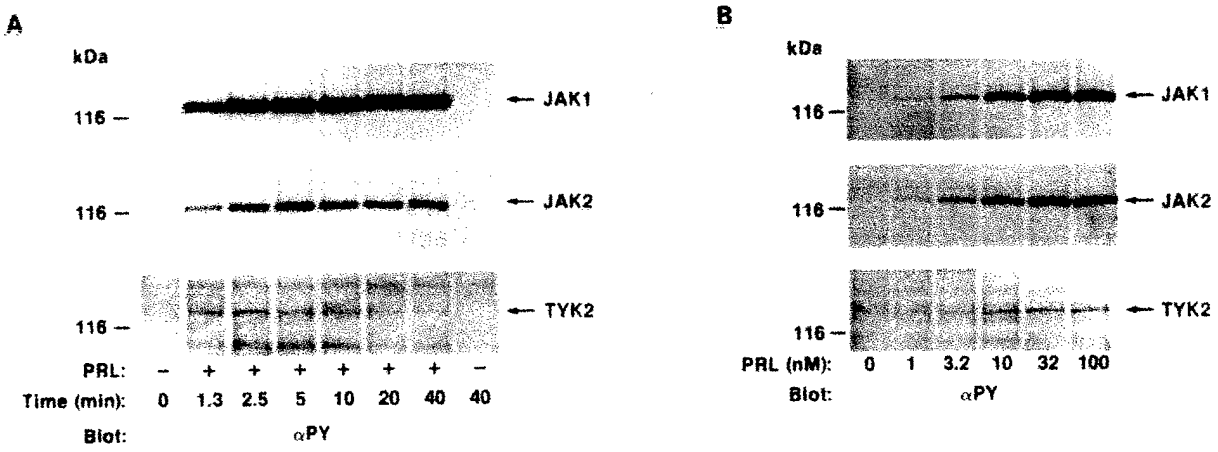

Fig. 2 PRL-induces tyrosine phosphorylation of Jak1, Jak2 and Tyk2 in T47D cells with similar time-kinetics and dose-responses: Antiphosphotyrosine immunoblots of immunoprecipitated Jak1, Jak2 and Tyk2 from lysates of T47D cells. The cells had been incubated with 100 nM PRL for varying times up to 40 min at 37 °C (Panel A), or with varying concentrations of PRL up to 100 nM for 5 min (Panel B). Molecular weight markers are indicated.

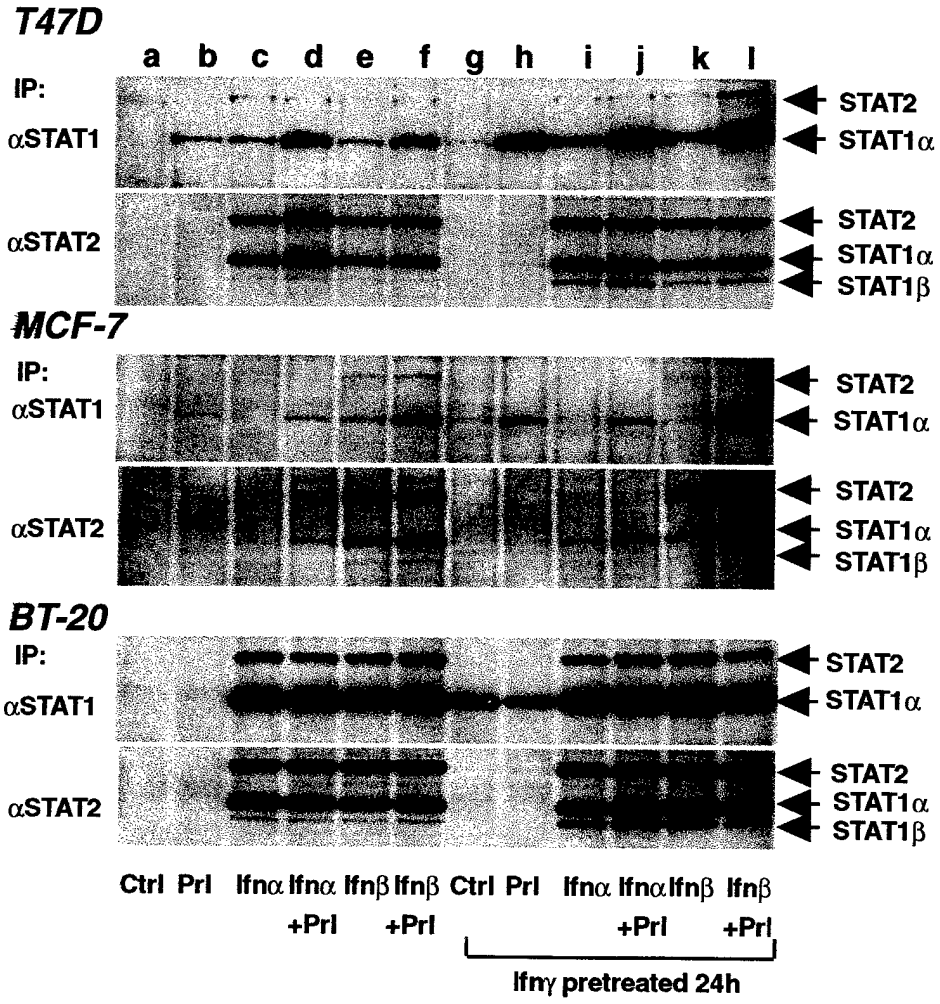

Fig. 3 Prolactin and interferon-α/β induced tyrosine phosphorylation of Stat1 and Stat2 transcription factors in human breast cancer cells: Effect of pretreatment with interferon-γ. Antiphosphotyrosine immunoblots of Stat1 and Stat2 immunoprecipitated (IP) from lysates of T47D cells (top panel), MCF-7 cells (middle panel) and BT-20 cells (lower panel). The cells had been preincubated for 24 h in serum-free medium in the absence or presence of human IFN-γ (10 ng/ml), before being stimulated for 15 min at 37 °C with medium (Ctrl), PRL (100 nM), IFN-α (1000 U/ml), IFN-β (100 U/ml), PRL+IFN-α, or PRL+IFN-β as indicated. Note that IFN-α/β and PRL-induced Stat1 tyrosine phosphorylation in general was additive. Furthermore, IFN-γ pretreatment augmented IFN-α/β induced tyrosine phosphorylation of Stat1β, particularly in T47D and BT-20 cells.

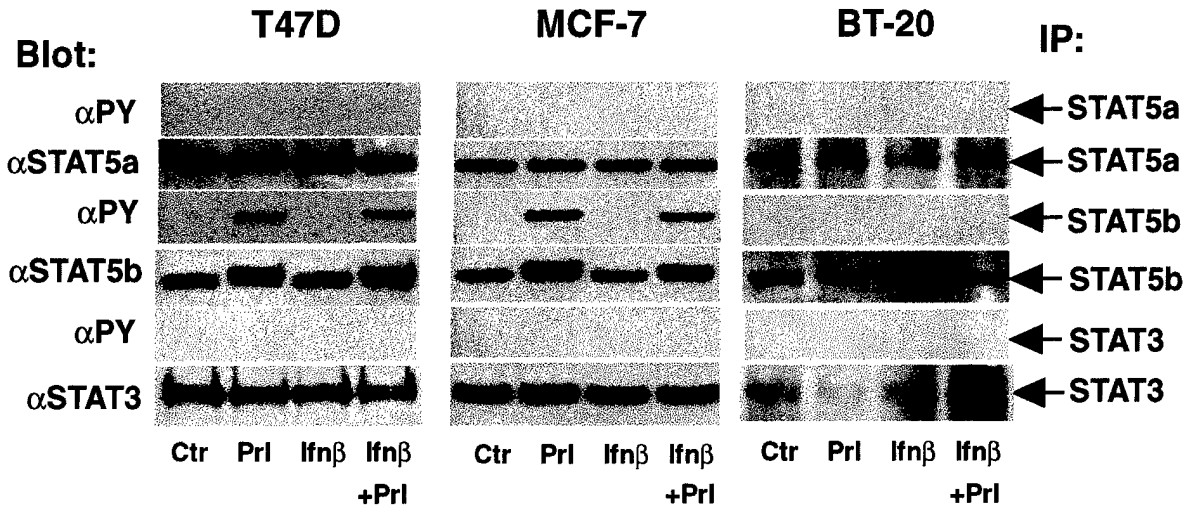

Fig. 4. Prolactin and interferon-β induced tyrosine phosphorylation of Stat5a, Stat5b and Stat3 transcription factors in human breast cancer cells. Antiphosphotyrosine (αPY) immunoblots and corresponding anti-Stat immunoblots of Stat5a, Stat5b and Stat3 immunoprecipitated (IP) from lysates of T47D cells (left panel), MCF-7 cells (middle panel) and BT-20 cells (right panel). The cells had been preincubated for 24 h in serum-free medium before being stimulated for 15 min at 37 °C with medium (Ctrl), prolactin (Prl; 100 nM), IFN-β (1000 U/ml), or PRL plus IFN-β as indicated. Note that PRL activates Stat5b, but not Stat5a in T47D and MCF-7 cells. IFN-β did not stimulate tyrosine phosphorylation of either Stat5a/b or Stat3 in these cell lines.

(7) CONCLUSIONS:

Repeated analyses have confirmed that a cytoplasmic protein, which is a negative regulator of transcriptional activation by IFN-α/β, human breast cancer cells, corresponds to a ~20 kDa polypeptide purified from human cervical carcinoma cells and designated *TKO* (transcriptional knock-out factor). Chromatographic purification of the material has been refined and western transfer from 2D gels improved to maximize retrieval of protein for mass spectrometric analysis. Peptide analysis and amino acid sequence analysis of proteolytic fragments from the highly purified material has been frustratingly delayed by technical problems or personnel transfers. Work now is in actual progress at the laboratory of Dr. Ruedi Aebersold, at the University of Washington in Seattle where there is unparalleled expertise in mass spectrometric technology. We can now realistically expect that it will be feasible to generate and pilot test monoclonal or polyclonal antibodies per original statement of work.

Tests of PRL signaling in three PRL-responsive human breast carcinoma lines (T47D, MCF-7 and BT-20) have indicated that Stat1 activation following PRL treatment is cell-dependent: differences in Janus kinase activities and Stat phosphorylations were observed in cells from well differentiated, estrogen-receptor-positive malignancies and cells from a poorly differentiated malignancy. PRL can differentially activate at least three distinct Jak tyrosine kinases in different human breast cancer cell lines, including Jak1, Jak2 and Tyk2. Initial findings support an hypothesis that changes in cytokine or growth factor signalling may regulate or reflect neoplastic evolution. Cell dependent differences may pertain to individualization of patient treatment protocols. Further work will continue to analyze differences in pathways of transcriptional activation by Stat3 and Stat5 and involve assessment of Stat DNA binding functions in EMSA using oligonucleotide probes representing the promoter elements of major interest to the antiproliferative pathway. These will include an IR sequence to represent the IRF-1 promoter critically implicated in IFN-α/β biologic activities.

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